EFFECTS OF GLUTATHIONE DEPLETION ON LEYDIG CELL AGING AND STEROIDOGENESIS

by
Lanre Adekola

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Abstract

Most aging men and many young men have reduced circulating testosterone levels, referred to as hypogonadism. Hypogonadism is estimated to affect about 5 million American men, including both aged and young. Low testosterone can have negative effects on mood, cognition, cardiovascular health, energy, libido, muscle mass, and bone density. Leydig cells are the testicular cells responsible for testosterone biosynthesis. A number of cellular changes have been identified in the steroidogenic pathway of aged Leydig cells that are associated with reduced testosterone formation, including reductions in luteinizing hormone (LH)-stimulated cAMP production, the cholesterol transport proteins steroidogenic acute regulatory (STAR) protein and translocator protein (TSPO), and downstream steroidogenic enzymes of the mitochondria and smooth endoplasmic reticulum. Many of the changes in steroid formation that characterize aged Leydig cells can be elicited by the experimental alteration of the redox environment of young cells, suggesting that changes in the intracellular redox balance may cause reduced testosterone production. As yet, cause-effect relationships have not been established, however.

Reduced glutathione (GSH), the most abundant intracellular small molecule thiol present in mammalian cells, serves as a potent intracellular antioxidant and is particularly abundant in Leydig cells. GSH decreases significantly as Leydig cells age. Given the abundance of GSH in Leydig cells, we hypothesized that the experimental depletion of GSH would result in an increasingly pro-oxidant intracellular environment, and that this would cause reduced steroidogenic function. Buthionine sulfoximine (BSO), a specific γ-glutamylcysteine synthetase inhibitor, can block the rate-limiting step of GSH biosynthesis, and by doing so deplete the intracellular GSH pool in both cultured cells and whole animals. To test the effect of decreased GSH on steroid formation, we first cultured MA-10 Leydig cells with BSO short-term (24 hours). This treatment reduced intracellular levels of GSH somewhat but had no effect on progesterone production by these cells at this early time. Nor did treatment of the cells with tert-butyl hydroperoxide (t-BuOOH, 2 hours), an oxidant. However, when the GSH-depleted cells

subsequently were exposed acutely to t-BuOOH, intracellular reactive oxygen species concentration was significantly increased, and this was accompanied by reductions in steroid production. These results suggested that alteration of the intracellular redox environment can result in the increased sensitivity of MA-10 cells to oxidative stress under circumstances in which the cells have reduced antioxidant capacity. In a second series of studies, cells were cultured for days to weeks with BSO alone. Over time, at times after GSH was reduced, steroid synthesis decreased, reminiscent of natural aging of primary Leydig cells. These results suggest that, as in aging, exposure to an increasingly pro-oxidant environment, over time, can have a negative impact on Leydig cell steroidogenic function, and that, indeed, increases in oxidative stress contribute to or cause the reduced testosterone production that characterizes Leydig cells aging. As of yet, the molecular mechanisms by which aging results in an altered redox environment remains unclear. Also uncertain is the molecular mechanism by which reductions in GSH and other antioxidant molecules in Leydig cells, and thus increased oxidative stress, elicit reduced sensitivity to LH and thus reduced steroid formation.

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Introduction

Many males experience significant decreases in serum testosterone levels by their fourth or fifth decade of life, clinically referred to as hypogonadism [1, 2]. Primary hypogonadism occurs when Leydig cells, the testosterone producing cells of the testis, fail to respond adequately to luteinizing hormone (LH) from the anterior pituitary gland to initiate steroid synthesis [3, 4]. Studies have suggested that this is in large part due to altered LH receptor-G protein coupling at the Leydig cell plasma membrane [3, 4].

Although the mechanism by which age-related defects occur remains uncertain, there is evidence that changes in the redox balance within the Leydig cells are involved. For example, numerous studies have suggested that imbalance of prooxidants and antioxidants within cells can lead to DNA, protein and/or lipid damage, and thus to functional changes [5, 6]. Cells produce reactive oxygen species (ROS) during normal metabolism. In steroidogenic cells, ROS production would be expected to be particularly high because in addition to the mitochondrial electron transport chain, steroid hydroxylations by cytochrome P450 enzymes produce ROS [7, 8]. This may have significance for Leydig cell function because of the detrimental effects that ROS can have on critical components of the steroidogenic pathway [9-11]. As Leydig cells age, the antioxidant defense molecules superoxide dismutase-1 and -2, glutathione peroxidase, and glutathione (GSH) are significantly reduced [12, 13]. Additionally, the superoxide content of aging Leydig cells is significantly increased compared to young Leydig cells [14]. Lipid peroxidation also increases, perhaps as a consequence of changes in the redox environment [12]. These results, though correlative, suggest that alteration in the redox environment of aged Leydig cells may be involved in the reduced testosterone formation that characterizes these cells.

Because GSH is so integrally involved in maintaining the cellular redox environment, its role in Leydig cell aging is of high interest [12]. It is our belief that there exists a correlation between diminished levels of GSH and diminished steroid synthesis in aged Leydig cells, and that oxidative stress induced by an improperly maintained redox environment is the chief factor

behind reduced LH receptor coupling sensitivity. The primary goal of this thesis is to determine precisely the effects an altered redox environment with reduced levels of GSH can have on Leydig cell health, measured as a function of steroid synthesis. A previous study of *Nrf2* knockout mice studied the effects of removing all major antioxidant pathways in mice on their Leydig cell antioxidant capacity and steroidogenesis [15]. It was found that *Nrf2*$^{-/-}$ mice experienced increased levels of oxidative stress at a 3 months (early age), which was followed by a significant drop in serum testosterone levels at 8 months (middle age) as opposed to 24 months (old age) in wild type mice, indicating that reduced antioxidant capacity precedes a decline in steroidogenesis [15].

While these data are crucial to understanding the role of oxidative stress in Leydig cell aging, there is a redundancy of roles between many of the antioxidants *Nrf2* regulates, and not all antioxidants involved in the *Nrf2* pathway are diminished with age [12, 15]. The experiments that were undertaken here were designed to distinguish GSH as a key factor in preventing the events that lead to Leydig cell decline. In order to do so, we conducted two sets of experiments using MA-10 cells, a mouse tumor Leydig cell line that produces progesterone rather than testosterone. These cells are simple to culture and monitor [16]. In a first set of experiments, we attempt to discern the effects of GSH depletion followed by short-term exposure to the oxidant t-BuOOH on steroid synthesis. The idea behind this is that acute exposure of cells with an altered redox environment to a stressor may mimic the effects of aging in several regards. In a second set of experiments, we attempt to observe the effects of long-term GSH depletion on steroid synthesis, another model for Leydig cell aging. It is hypothesized that MA-10 cells will be highly susceptible to oxidative stress and diminished steroid synthesis following GSH depletion and also that long-term GSH depletion will precede a decline in steroid synthesis, similar to what apparently occurs *in vivo*.

LITERATURE REVIEW

Background

Testosterone is a steroid hormone recognized for its role in masculinization of the body as well as its support of spermatogenesis. It is the primary male sex hormone and targets a diverse array of cell types throughout the body. It is first synthesized by males in utero, and is essential for the development of external genitalia and sex characteristics, both directly and indirectly through the actions of its metabolites [3, 17]. In the adult body it has a wider range of roles but is chiefly involved in virilization.

Testosterone elicits numerous physiologic and mental effects, some potent and long term such as the induction of puberty and male development, and others more transient and short term such as regulating libido and sexual activity. Changes in both interstitial and serum testosterone are associated with numerous health states and conditions that can have an impact on fertility, metabolism, lean muscle mass, and psychological state [18-20]. Numerous studies also exist assessing the possibility for an impact of testosterone on cardiovascular health, mental health and neurodegenerative disease, prostate health, gut biome composition, and the immune system [21-25].

As aging occurs in the adult male, a noticeable decrease in serum and intra-testicular testosterone occurs, likely due to a lifetime of wear brought upon the Leydig cells of the testis through the process of steroidogenesis [4, 26]. Steroidogenesis is a highly ATP consumptive process and also involves heavy use of cytochrome p450 enzymes [27]. The combination of these two factors over a lifetime may put Leydig cells at elevated risk for oxidative damage, ironically affecting their ability to synthesize steroid. As a consequence, a significant population of adult males experience a decline in testosterone levels by their fourth or fifth decade of life and are either subject to or at high risk for obesity, osteoporosis, frailty, fatigue, erectile dysfunction, depression, and general feelings of malaise [28]. This state of having low testosterone is referred to as hypogonadism, and the vast majority of males experience it to some extent as they age.

Steroidogenesis

Testosterone is a lipid hormone, synthesized through a series of reactions regulated by the hypothalamic-pituitary-gonadal axis. The hypothalamus has the capacity to assess levels of circulating serum testosterone and is activated by low concentrations to signal to the pituitary gland. Neurons of the hypothalamus secrete gonadotropin-releasing hormone (GnRH) through the portal vasculature system that connects it to the anterior pituitary gland, thus inducing it to secrete both follicle-stimulating hormone (FSH), which is involved in spermatogenesis, and luteinizing hormone (LH), which sets steroidogenesis into motion [1, 29, 30].

Upon release from the anterior pituitary into the bloodstream, LH reaches the Leydig cells of the testis and acts on their surface receptors. Leydig cells possess the cellular machinery required of steroidogenesis, and are the primary producer of testosterone in the male body [27]. They arise from a stem cell found in the interstitial compartment of the testis [3, 31]. This cell is undifferentiated and is thought to have the capacity for asymmetric division to produce both another stem cell and the first cell in the Leydig cell lineage, the progenitor Leydig cell. These cells are competent to produce large amounts of testosterone metabolites, but not testosterone itself [3, 31]. The progenitor Leydig cells differentiate into immature Leydig cells, and the latter into adult Leydig cells at the onset of puberty; the adult cells produce testosterone as their major product and reside in the interstitial compartment of the testis between tubules [31, 32].

Leydig cells contain G-protein-coupled receptors (GPCR) on their cell surface that are involved in the initiation of steroidogenesis. The receptor itself is a seven-pass transmembrane protein that associates with a G-protein. The G-protein is hetero-trimeric, consisting of three subunits: alpha, beta, and gamma [33]. The beta and gamma subunits are typically found bound together, and the alpha subunit interacts with the G-protein, the beta-gamma subunits, and a molecule of GDP when in its inactive form [4, 33, 34].

Many of the mechanisms behind steroidogenesis and the down stream effects of LH-receptor coupling can be observed in Figure 1. Ligand binding activates the receptor and the

alpha subunit, initiating an exchange of the bound GDP for GTP. The Alpha subunit then dissociates from the receptor and beta-gamma subunit to activate the enzyme adenylyl cyclase [34]. Upon activation, adenylyl cyclase functions to convert ATP to cyclic AMP (cAMP), a secondary messenger molecule that triggers further downstream effects of the steroidogenic pathway [33, 35]. As long as the activated alpha subunit remains bound, adenylyl cyclase will remain active. Over time, inherent GTPase properties of the alpha subunit will convert the bound GTP to GDP thus shutting off the pathway [4, 33, 34].

Adenylyl cyclase activation and cAMP generation activate protein kinase A, an enzyme that initiates a signaling cascade that in turn activates CREB, a transcription factor that is involved in Leydig cell function [36]. cAMP also facilitates steroidogenesis by aiding in the import of cholesterol into the mitochondria, which is the rate-determining step of the pathway [37, 38]. Because free circulating cholesterol is toxic, it is often stored in the plasma membrane in an esterified form [33, 39]. cAMP acts by phosphorylating and activating hormone-sensitive lipase (HSL), an enzyme that cleaves cholesterol-esters into free cholesterol so that it may then be used for testosterone synthesis [33]. Like cAMP, HSL is also involved in the protein interactions that then bring cholesterol into the mitochondria [40].

Prior to entering the mitochondria, cholesterol interacts with both Translocator Protein (TSPO) and Steroidogenic Acute Regulatory protein (StAR) to gain entry into the inner mitochondrial membrane so that the enzymatic processes of steroidogenesis can initiate [33, 41, 42]. Evidence suggests that StAR is responsible for binding free cholesterol outside the mitochondria and transferring it to the outer membrane where it then binds TSPO, which transfers it to the inner membrane [35].

In the inner membrane, cholesterol is converted into pregnenolone by CYP11A1, a cytochrome P450 enzyme that cleaves the C-27 side chain of the cholesterol molecule [37]. Once generated, pregnenolone is translocated to the smooth endoplasmic reticulum to undergo further enzymatic modification. 3-Beta Hydroxysteroid Dehydrogenase (3b-HSD) converts pregnenolone

into progesterone, after which progesterone is subsequently converted into 17α-hydroxyprogesterone by the enzyme 17α-hydroxylase. 17α-hydroxyprogesterone is then enzymatically modified by C17-20 lyase to yield the androgen androstenedione. The final step in steroidogenesis is the conversion of androstenedione into testosterone by 17-ketosteroid reductase [43]. Leydig cells then excrete testosterone where it can act on nearby Sertoli cells within the seminiferous tubules to support spermatogenesis or can enter the blood stream and in this way can reach and act on a wide variety of effector cells.

High levels of circulating testosterone feed back negatively on the hypothalamus and anterior pituitary gland to inhibit release of GnRH, FSH, and LH. This in turn shuts down steroidogenesis, which may only be reactivated once testosterone levels are sufficiently low [25, 41]. Accordingly, pulsatility of LH is key for maintaining proper steroidogenesis, and several studies suggest that improper LH pulsatility is associated with aging and poor steroid synthesis [30, 35, 44].

Testosterone synthesis however is not entirely limited to the Leydig cells of the testis. Steroid hormones are produced with high frequency in the adrenal cortex of the adrenal gland, and due to shared enzymes between these pathways, up to 10% of all circulating testosterone in adult males result from conversion of adrenal steroids, such as aldosterone [45]. This is highly important due to the potential for impact on androgen sensitive diseases, such as prostate cancer [45].

The Effects of Testosterone on the Body

Because testosterone, as well as other steroid hormones, is lipophilic, it is capable of passing through cell membranes into the cytoplasm with ease where it can then act on the androgen receptor [17]. The androgen receptor is a member of the nuclear receptor superfamily and exists inactive and bound to heat-shock chaperone proteins prior to activation [46].

Structurally, the receptor is a protein that contains a ligand binding domain specific for androgens, a DNA binding domain that recognizes the nucleotide sequences of androgen responsive elements, and an amino-terminal activation domain [46].

Upon the binding of testosterone or other androgens to their receptor, the chaperone proteins dissociate. Activated and phosphorylated, the ligand bound androgen receptor translocates to the nucleus where it forms a homodimer that serves as a transcription factor and triggers gene expression and protein synthesis [46]. Depending on the particular stage of life and target tissue, testosterone can induce a wide range of effects. In utero testosterone exposure is key for the development of the male body [47]. Prior to any major sexual differentiation, the fetus has a bi-potential gonad that can develop into either the male or female reproductive tract, and eventually the SRY gene of the Y chromosome in males encodes for fetal Leydig cell development [17]. These cells then produce testosterone that promotes the formation of the Wolffian duct, which eventually becomes the testis [17, 47]. Furthermore, testosterone can be converted by the enzyme 5a-reductase into Dihydrotestosterone (DHT), a more potent androgen specific to tissues that do not respond to testosterone, and promotes development of the prostate and urethra, as well as the masculinization of external genitalia [17].

This relationship between androgen action and masculinity continues again in puberty, where sudden spikes in testosterone synthesis induce virilization. During puberty, increased GnRH and thusly LH pulsatility begin, and androgens mediate the development of secondary sexual characteristics that are typified by muscle, hair, and overall body growth [28, 30].

The effects of androgens on muscle are perhaps their most known and pronounced activity in the body. Testosterone is a regulator of many metabolic processes, and causes dose-dependent increases in skeletal muscle mass and volume, fat-free body mass, strength, and power [48, 49]. Studies suggest that it is also directly responsible for increases in Insulin-like Growth Factor 1 (IGF-1), hemoglobin and erythropoiesis, concentration of high-density lipoprotein cholesterol,

and sexual desire and activity [48]. Conversely, low levels of testosterone have been associated with a reversal of the aforementioned trends.

Several concerns with regard to testosterone exist surrounding potential negative effects on cardiovascular and prostate health. Because circulating testosterone can be converted to DHT that acts on the prostate, concerns have been voiced over the ability of testosterone to increase prostate specific antigen (PSA) secretion as well as induce benign prostatic hyperplasia (BPH) [28, 48].

Testosterone Regulation of Spermatogenesis

The creation and development of viable gametes in the testis is a dynamic and lengthy process that is initiated at the onset of puberty and is continuous throughout the life of all healthy adult males. Early spermatogenesis takes place within the epithelial lining of the seminiferous tubules of the testis, with millions of sperm developing in syncytium [50]. Sperm develop from progenitor stem cells called spermatogonia, which are capable of dividing through mitosis to produce both a copy of themselves and cells that enter a differentiation pathway [50]. Differentiation of these cells results in the entry of cells into meiosis during which primary spermatocytes divide to form two secondary spermatocytes which then undergo meiosis II to yield four spermatids that can then differentiate into sperm [50].

This entire process occurs on the basal-lamina-lumen axis of the tubules, with more immature cells found at the walls of the tubules and mature sperm well into the interior. These sperm then exit the lumen of the seminiferous tubules and enter the epididymis where they develop and gain the capacity for fertilization [50].

The presence of FSH and intra-testicular testosterone are crucial for the proper maintenance of spermatogenesis. FSH is involved in regulation of spermatogenesis through its actions on Sertoli cells, somatic cells of the seminiferous tubules around which immature sperm

are supported and developed [50]. Studies show that inhibition of FSH and LH have a negative impact on spermatogenesis, and can induce Azoospermia, the absence of viable sperm [29].

Because Leydig cells lie in the interstitial space in between the seminiferous tubules, when testosterone reaches the cells of the testis that contain androgen receptors such as Sertoli cells, peritubular cells, and Leydig cells themselves, the concentration is much higher than in any other region of the body [29]. In fact, intra-testicular testosterone concentration is approximately 30 times greater than in serum, and the lower limit of testosterone required to sustain spermatogenesis is still over ten times greater than serum levels [29]. Once below this limit however, apoptosis of spermatocytes is induced due to lack of androgen receptor nuclear localization and the subsequent gene expression that maintains it, thus indicating that spermatogenesis is an androgen-dependent process [29, 38].

Although much is unknown about the specific actions of androgens on Sertoli cells to support spermatogenesis, it is known that the relationship between Sertoli cell androgen receptor expression and testosterone concentration is unique. Studies using the Brown Norway rat as a model organism have shed tremendous light on the processes of Leydig cell function and aging due to strikingly similar paths of development and senescence between it and humans [5]. Studies involving this rat have observed that decreased levels of testosterone down-regulate Sertoli cell nuclear androgen receptor expression but have no effect on androgen receptor mRNA levels. This implies that a major role of testosterone in the testis is regulating the translation of the androgen receptor, which eventually induces spermatogenesis [38].

Hypogonadism

Male hypogonadism is the state of having low serum testosterone, which in turn can be detrimental to mental health, metabolic processes, and sexual function among many other issues. It can also lead to impairment of spermatogenesis, which manifests itself as azoospermia or oligospermia. It is perhaps most commonly attributed to aging, but can also be caused by injury

or congenital defects that include but are not limited to: metabolic deficiencies, gene mutations, improper migration of kisspeptin neurons, exposure to phthalates and endocrine disruptors, and Androgen Insensitivity Syndrome [17, 30, 41].

Regardless of its source, hypogonadism manifests itself in two forms – primary and secondary – that both feature major disruptions of the hypothalamic-pituitary-gonadal axis and either induce low serum testosterone or make the body incapable of responding to it whatsoever. Primary hypogonadism occurs due to errors of the testis, where Leydig cells are incapable of properly responding to LH signaling. When a consequence of aging, testosterone is still synthesized, but in significantly diminished amounts. Secondary hypogonadism features irregularities of the hypothalamus and pituitary gland where GnRH or LH is not released in adequate amounts in response to low serum testosterone. Often as a result of low intratesticular testosterone, developing sperm are deprived of the signaling required to support spermatogenesis, resulting in subsequent infertility. Hypogonadism is a serious public health issue and a major medical concern for those whose quality of life has been significantly impacted as consequence of low testosterone, as well as young hypogonadal men that desire to father children. Consequently, large amounts of resources are being used to understand, treat, and prevent it.

The Effects of Leydig Cell Aging on Male Reproductive Health

Many of the health issues and symptoms attributed to aging such as increases in adiposity and decreases in energy, mood, muscle mass, libido, and even cognitive function, have a strong association with decreases in serum testosterone. While it is certain that aging takes a toll on steroidogenesis, whether or not these symptoms are caused by decreased serum androgens or are simply correlated to them remains unclear. What has been elucidated however, is that there is no significant loss of LH from the anterior pituitary gland with age, as serum LH either increases or remains unchanged over time [35]. This is peculiar because it implies that the loss of steroidogenic capacity is solely due to Leydig cell dysfunction.

More studies using the Brown Norway rat have demonstrated that stimulating aged males with exogenous LH is not sufficient to increase serum testosterone levels to that of a young male [35]. As a result of these findings, a large amount of research has gone into understanding the differences between young and old Leydig cells, as well as which steps in the steroidogenic process are susceptible to breaking down with age. It was found that stimulating Leydig cells with cAMP was able to restore serum testosterone to youthful levels. Since cAMP is only involved in steroidogenesis upon the activation of the LH receptor, this finding indicates that not only does decreased Leydig cell sensitivity stem from the inability to respond to LH signaling, but also that this step can be bypassed [35]. Further analysis of LH signaling has revealed both a deficiency in the ability of the LH receptor to activate adenylyl cyclase and of cholesterol to translocate into the inner mitochondrial membrane. The latter issue is likely related to observed decreases in TSPO and StAR in aged Leydig Cells [35].

The involvement of cyclooxygenases has also been linked to the decline of testosterone synthesis in Leydig cells [3]. Cyclooxygenases are enzymes that function to metabolize arachidonic acid in cells, and Cyclooxygenase-2 (COX2) in particular has also been found to be inhibitory of steroidogenesis [51, 52]. Because LH signaling and subsequent cAMP production trigger the release of arachidonic acid in Leydig cells, COX2 protein expression steadily follows LH stimulation [3, 52]. Although the concepts behind LH signaling inducing both steroidogenesis and the COX2 expression that serves to inhibit it are conflicting in nature, Brown Norway rats have been shown to exhibit heightened amounts of COX-2 with age, thus suggesting it as another possible cause of age-induced decline in serum testosterone [52].

Leydig Cell Aging and Oxidative Stress

While much is yet to be discovered regarding the source of the malfunction of the proteins integral to steroidogenesis, researchers have attempted to form a link between the decline of Leydig cells and the ability of reactive oxygen species (ROS) to induce cellular damage [53].

ROS are oxygen based molecules that either possess a free radical or are a minor reaction away from doing so, such as hydrogen peroxide, superoxide, and free hydroxyl radical [4]. ROS have a unique penchant for inducing chain reactions that strip molecules of electrons and can induce doubles strand breaks in DNA that damage it beyond repair. ROS also induce lipid peroxidation and protein damage, and the accumulation of their actions have been known to trigger many of the symptoms of aging such as loss of cell function, cell death, and even cancer [4, 53].

The vast majority of intercellular ROS are generated as a byproduct of the electron transport chain during oxidative phosphorylation [53]. The electron transport chain is a series of protein complexes located about the mitochondrial membrane that uses successive redox reactions to convert molecular oxygen into energy for the cell, primarily in the form of ATP [53]. As oxygen traverses the electron transport chain, there are two major points in which ROS generation can occur: Complex I and Complex III. These complexes feature quinonoids and flavonoids, electron and proton transferring molecules of the mitochondria that are heavily responsible for the incidental generation of ROS [50].

In complex I, NADH transfers 2 electrons through a series of iron-sulfur clusters to ubiquinone, yielding ubiquinol. Slippage of electrons here generates semiquinone, a radical ion of ubiquionol that can convert O_2 to O_2^- (superoxide). In Complex III cytochrome b reduces a molecule of ubiquinone to ubiquinol, one electron at a time, with semiquinone radical anion formed temporarily as an intermediate in the process [53, 54, 55].

Slippage of electrons and superoxide synthesis occur so infrequently that superoxide accounts for less than 1% of electron transfer from NADH to oxygen [54, 56]. Nevertheless, even a small amount of superoxide has the potential to initiate chain reactions that damage DNA. Superoxide contributes further to potential oxidative stress through its deactivation of aconitase, succinate dehydrogenase, and NADH-ubiquinone oxidoreductase [50], which are enzymes in the transport chain that contain iron-sulfur clusters among other metals. This deactivation frees iron and or copper ions for use in the Fenton reaction to generate free hydroxyl radical [55]. In the

Fenton reaction, hydrogen peroxide is reduced by iron or copper ions to yield two hydroxyl radicals. Hydroxyl radical is significantly more dangerous to the cell than superoxide and can incur substantial peroxidative damage to proteins, lipids, and DNA.

A typical cell however has multiple mechanisms in place to remove ROS. Superoxide dismutases (SOD) are a family of enzymes that are essential for the removal of superoxide [57, 58]. They catalyze the conversion of superoxide to hydrogen peroxide, a much more stable and less reactive molecule. Glutathione peroxidase can then catalyze the reduction of hydrogen peroxide to water by reduced glutathione (GSH). Likewise, catalase can enzymatically convert hydrogen peroxide to water and oxygen [57, 58]. Without these antioxidants, hydrogen peroxide produced from SODs may be subjected to the Fenton reaction.

The free radical theory of aging suggests that despite these many mechanisms of ROS removal, repeat and acute exposure over a lifetime facilitates the degradation of the cell [12, 53, 54, 55]. This is especially relevant to Leydig cells because steroidogenesis is a highly ATP consumptive process. Much data exist demonstrating a correlation between ATP and testosterone synthesis in Leydig cell mitochondria [14, 27]. While inhibition of the mitochondrial electron transport chain produces a markedly large drop in the generation of ATP, it is also shown to suppress testosterone synthesis, implying that the electron transport chain is the driving force behind steroidogenesis rather than glycolysis [27]. Cells that are dependent on glycolysis for energy rather than oxidative phosphorylation are likely to be exposed to lower amounts of ROS. Accordingly so, it is logical that a cell such as the spermatozoa, which is responsible for the passing on of genetic material and is also created from frequently dividing cells, would be inclined to use glycolysis as a means of energy [27]. Leydig cells however rarely turnover, and this may indicate their preference for a less safe but more efficient process for the generation of energy [3]

Leydig cells and other steroidogenic cells are at an elevated risk of experiencing oxidative stress and subsequent lipid peroxidation because of the free radicals they generate

during steroidogenesis in addition to oxidative phosphorylation [12]. It has been shown that free radicals inhibit steroid synthesis by interfering with cholesterol transport into the mitochondria as well as the functions of p450 enzymes [12, 59-64]. As expected, LH has been linked to increased amounts of ROS in Leydig cells. Furthermore, LH has also been found to be involved in activating the cellular response to oxidative damage in Leydig cells [4]. Stimulating rats with LH increases gene expression for numerous anti-oxidant pathways, particularly *Nrf2*, a transcription factor that is a master regulator of the cell's oxidative stress response pathway [4].

Further support of the effects of long-term wear on Leydig cell steroidogenesis is found in hormone suppression studies. Through use of Silastic implants, contraceptive amounts of testosterone were given to young rats to halt steroidogenesis and thusly put their Leydig cells in a state of dormancy [25]. After the removal of these implants, aged rats were found to synthesize testosterone at levels comparable to young rats, reaffirming that age related deficiency of testosterone is initiated by the long-term use of the cell's steroidogenic machinery [25]. While long-term suppression of LH in humans to prevent Leydig cell aging is impractical, these studies leave room for the idea that a diet or regimen heavily involving antioxidants has the potential to reverse or prevent the causes of Leydig cell aging.

A Cao et al. study on antioxidant expression in young and aged rat Leydig cells showed that aged Leydig cells, in comparison to young, had decreased expression of (GSH), while other non-enzymatic antioxidant levels (vitamin A, vitamin E, and oxidized glutathione) remained unchanged [12]. This suggests that there is an age related loss of GSH. Since oxidized glutathione (GSSG) levels remained unchanged, perhaps the balance between the two affects cellular susceptibility to oxidative stress. The same study found that enzymatic antioxidant SODs, glutathione peroxidase, and catalase were all also down in aged cells. GSH is integral to maintaining the cellular redox environment, where it serves as a reducing agent in the mitochondria while GSSG functions as an oxidizing agent. If the ratio of the two becomes heavily skewed in favor of GSSG, it can create an oxidizing environment that perhaps allows reactive

oxygen species to thrive and prevents their reduction to more stable, less harmful molecules [65, 66]. Furthermore, Glutathione is also involved indirectly in other antioxidant processes, such as maintenance of vitamins E and C in their reduced forms, where they are able to attenuate harm from ROS [67, 68]. Taken together, GSH depletion and poor maintenance of the cellular redox environment with age may be a driving factor of Leydig cell aging and loss of steroidogenic function.

The effects of oxidative stress on DNA are only one of many other factors that contribute to aging, including but not limited to telomere shortening and the build up of naturally occurring DNA mutations. Nevertheless, the potential for ROS to damage cells is so large and evident that oxidative stress is generally perceived as a major contributing factor for aging [12, 14, 34]. Lipid peroxidation induced by reactive oxygen species is of particular interest for Leydig cell aging because the LH receptor is located within the lipid membrane of the cell. Theoretically, lipid membranes rapidly turn over and the LH receptor should be continuously translated as necessary. Therefore, decreased sensitivity of LH receptor coupling and its ability to be bypassed suggest that if ROS are indeed responsible for decreased steroidogenesis in aged cells, they may be acting specifically in the lipid membrane damaging the LH receptor, or acting on the regions of DNA encoding the ligand binding region of the LH receptor. Careful observations of the mechanism(s) behind the regulation of the cellular redox environment as well as specificity of ROS for particular organelles is necessary to further understand the process of Leydig cell steroidogenic decline as well as aging at large.

Glutathione Structure, Synthesis, and Regulation

Glutathione is a tripeptide molecule consisting of glycine, cysteine, and glutamic acid. It is synthesized via two successive ATP dependent reactions by the enzymes gamma-glutamylcysteine synthetase and glutathione synthetase. It functions as a powerful reducing agent and is heavily involved in the maintenance of the cell's redox environment. Its main functions

within the cell are based upon the interactions of its thiol group (S-H), which has the ability to serve as a proton donor. It is through critical reactions concerning the donation and acceptance of protons and electrons that it is able to demonstrate its antioxidant capacity and assist in the regulation of cellular homeostasis. In the presence of ROS, such as hydrogen peroxide, the enzyme glutathione peroxidase catalyzes the conversion of hydrogen peroxide to water using GSH as a cofactor [69]. The reaction catalyzed by glutathione peroxidase is annotated as such: $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ [50].

Once GSH has donated its electron, it then possesses a free radical ion with the potential to damage molecules. To prevent such damage, one free radical ion possessing glutathione molecule can bind with another to form glutathione disulfide, the oxidized state of glutathione (GSSG) [50]. The enzyme glutathione reductase is then used to restore GSH, by catalyzing the reaction: $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$ [50, 70]. Up to 98% of glutathione exists in its reduced form, and it is the proper maintenance of the GSH:GSSG balance that determines a cell's ability to adequately respond to oxidative stress and in effect limit aging [66, 71]. The ratio of GSH:GSSG is a strong indicator of overall cell health [65]. A strong correlation exists between decreased levels of GSH and some disease states associated with aging such as Parkinson's disease and Alzheimer's disease [66]. An excess of glutathione can also be detrimental to cells, with heightened levels being associated with tumor cell resistance to alkylating agents. Potential treatment for this can be found through use glutathione inhibitors such as buthionine sulfoximine (BSO), a chemotherapeutic agent that inhibits glutamylcysteine synthetase and thus prevents glutathione synthesis [72].

Materials and Methods

Chemicals

L-buthionine-sulfoximine (BSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Waymouth's MB/752 medium, horse serum, and tert-butyl hydroperoxide (t-

BuOOH) were from Sigma-Aldrich (St. Louis, MO). Progesterone antibody was from ICN (Costa Mesa, CA). Progesterone was from Steraloids (Newport, RI). M-199 medium was from Invitrogen (Carlsbad, CA). Bovine LH (USDA-bLH-B-6) was provided by the U.S. Department of Agriculture Animal Hormone Program (Beltsville, MD). Testosterone was from Steraloids (Newport, RI).

MA-10 Cell Studies

The MA-10 mouse Leydig tumor cell line was a generous gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA). The cells were cultured in Waymouth's MB/752 medium containing 15% horse serum and 5% CO_2 at 34°C. Although Waymouth's MB/752 medium contains a relatively high concentration of GSH, BSO treatments in the present studies depleted intracellular GSH. MA-10 cells were plated onto 60 x 15 mm plates in Waymouth's MB/752 culture medium with 15% horse serum.

In the first set of experiments, cells were plated into 24 well plates. After 24 hours, 100μM BSO was added to the medium. After 24 hours of BSO incubation, the medium in control and BSO treated wells was removed for subsequent treatment of the cells with t-BuOOH and LH. The cells were assayed for GSH by methods outlined below.

Control and BSO treated cells were incubated with maximally stimulating LH (10ng/ml) *and* tert-butyl hydroperoxide (t-BuOOH) of increasing concentrations (0μM, 6.25μM, 12.5μM, 25μM, and 50μM) in DMEM/F12-M199 culture medium with .01% BSA. M-199 medium is a cholesterol-containing medium that contains low GSH. After 2 hours, the medium was collected for the purpose of progesterone assays (RIA). Media from 4 different experiments were analyzed for each treatment. Next, both groups (i.e. control and BSO- treated) were cultured with maximally stimulating LH (10ng/ml) *alone* in DMEM/F12-M199 culture medium. After 1 hour, the medium was collected for progesterone assays. Media from 4 different experiments were

analyzed for each treatment. The cells were frozen in -80°C, and assayed for MTT reduction (cell viability). Another set of cells treated with BSO/t-BuOOH as above were allowed to undergo a 48 hour period of recovery in Waymouth's MB/752 medium containing 15% horse serum and 5% CO_2 at 34°C, after which they were cultured with maximally stimulating LH (10 ng/ml) in DMEM/F12-M199 culture medium for 2 hours. Some cells were frozen (80°C) and others assayed for MTT reduction. Media from 4 different experiments were analyzed for progesterone for each treatment.

In another set of experiments, cells were cultured long-term in the presence of BSO. Every four days for four weeks, cells were treated with one of three concentrations of BSO (25 µM, 50µM or 100 µM). 24 hours after BSO treatment, cell samples were both collected for GSH analysis and plated into a 24 well plate where they were incubated with maximally stimulating LH (10ng/ml) alone in DMEM/F12-M199 culture medium. After 1 hour, the medium was collected for progesterone assays. Progesterone concentrations were determined by radioimmunoassay (RIA) and expressed as nanograms/ml culture medium.

GSH assay

Cells were lysed in 5% metaphosphoric acid and sonicated. Protein was precipitated by centrifugation at 13,000g for 30 minutes. The supernatant was diluted 10 fold with sodium phosphate buffer (0.1M, pH-8.0, with 5mM EDTA). Diluted samples (10µl) were incubated with 10 µl of o-phthalaldehyde (in methanol) and 180 µl of phosphate buffer for 15 min at room temperature. Fluorescence was read with a BioRad luminescence spectrometer (excitation wavelength 350nm and emission wavelength 420nm). The cellular GSH content was calculated using a GSH standard curve that was run concurrently. Cells from 4 different experiments were analyzed for each treatment.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). For two group comparisons, Student's t-test was used. For multi-group comparisons, group means were evaluated by two-way ANOVA. If group differences were revealed by ANOVA ($P<0.05$), differences between individual groups were determined with the Tukey's test using SigmaStat software (Systat Software Inc., Richmond, CA). Values were considered significant at $P<0.05$.

RESULTS

Effects of short-term BSO treatment on intracellular GSH and steroidogenesis in MA-10 cells

In order to demonstrate the effects of short-term BSO treatment on intracellular GSH levels, MA-10 cells were incubated with increasing concentrations of BSO in culture medium (25μM, 50μM, 100μM) for 24 hours. Figure 2A shows concentration-dependent decreases in intracellular GSH with increasing amounts of BSO when compared to control cells. BSO at 25μM produced a 22% decrease in GSH compared to control, with 50μM and 100μM producing 28% and 33% decreases, respectively. Following 24-hour BSO treatment, cells were washed free of BSO and incubated with maximally stimulating LH (10ng/μl) for 2 hours. Figure 2B shows no major differences in progesterone formation and thus no effect of short-term (24 h) BSO treatment.

Effect of an oxidizing agent on steroidogenesis in an altered redox environment

To determine whether an altered redox environment resulting from reduction in GSH would sensitize the cells to subsequent exposure to an oxidant, MA-10 cells first were cultured in 100 μM BSO for a 24 hour period. Cells cultured with BSO for 24 hours were then cultured for 2 hours with increasing concentrations of t-BuOOH (0μM, 6.25μM, 12.5μM, 25μM, or 50μM) and with maximally stimulating LH. As also shown in Figure 2B, when cells were cultured in the absence of t-BuOOH, there was no difference in progesterone produced between control cells and

cells treated with BSO for 24 hours (Fig. 3). However, as also shown in Figure 3, when control and BSO-treated cells were incubated with 6.25μM t-BuOOH, both the control and BSO-treated cells showed decreases in progesterone production compared to control cells that did not receive t-BuOOH. At 12.5μM t-BuOOH, progesterone production decreased by both BSO pretreated and the non-BSO treated controls. However, a significantly greater decrease in progesterone production was seen by the BSO-treated cells. This same pattern was true at 25 and 50 μM t-BuOOH; progesterone decreased more in cells that had been pre-treated with BSO.

Effects of continued BSO treatment on intracellular GSH and steroidogenesis in MA-10 cells

After observing that short term (24 hour) BSO incubation had little affect on progesterone production, but that BSO incubation followed by t-BuOOH caused a large decrease in progesterone, we sought to determine if long-term GSH depletion alone would have an effect on progesterone synthesis. In order to observe this, cells were treated with increasing amounts of BSO in culture medium (0μM, 25μM, 50μM, 100μM) for 24-hour periods every 4 days for 28 days. After each treatment, cells were assessed for GSH content and for their ability to produce progesterone in response to maximally stimulating LH for 2 hours. Figure 4 shows that as was the case for a 24-hour incubation with BSO, incubations with BSO over the course of 8 days had minor effects on GSH and also had no effect on progesterone production.

Figure 5 shows cells after incubations with BSO over the course of 12 days of culture. As seen in Figure 5A, cells treated with 25μM BSO showed a 40% decrease in GSH and a 26% decrease in progesterone (5B). Incubation of cells with 50μM BSO resulted in a 28% decrease in GSH and decreased progesterone output by 36%. Cells given 100μM BSO showed a 28% decrease in GSH and a 39% decrease in progesterone. Figure 6 shows cells after 28 days of BSO treatments. Decreases were seen in GSH, as at 12 days. Decreases in progesterone were 40% at 25μM BSO, and with 50 and 100 μM BSO, 45% and 29%, respectively.

Longitudinal effects of BSO incubation on GSH and progesterone synthesis

Figures 7, 8, and 9 concern the longitudinal effects of incubating cells in a given concentration of BSO on GSH, over time. Figure 7 shows changes in intracellular GSH levels of cells given 25µM BSO at each 4-day interval for 28 days. Figures 8 and 9 show the longitudinal effects of BSO treatment at 50µm and 100 µM respectively. Data are expressed both as GSH concentration (A) and as percent of control (B), where control cells were not given BSO but were cultured for the same amount of time as BSO treated cells. According to Figure 7, continued incubation of cells in 25µM BSO did not have a consistent effect on GSH levels, as concentrations varied between treatments without forming a trend. When compared to control cells, however, 25µM BSO cells consistently exhibited diminished levels of GSH. Similar results are seen in figures 8 and 9 for both 50µM and 100µM BSO treated cells; intracellular GSH levels varied in these cells throughout BSO incubations, but still were consistently lower than that of control cells.

Figure 10 shows changes in progesterone synthesized by cells given 25µM BSO at each 4-day interval for 28 days. While the concentration of progesterone appears to diminish from days 8-28, when observed as a percent of control these cells showed decreased levels of progesterone on days 12 and 28 compared to control, which remained throughout all subsequent BSO incubations. A similar trend is observed in figures 11 and 12, which show progesterone levels over time in cells cultured in 50 and 100µM BSO, respectively. Concentration of progesterone appears to decrease over time, but when observed as a percent of control there are only slight changes in progesterone from days 0-8. By 12 days of culture through 28 days however, there is a large decrease in progesterone in comparison to control cells.

Figure 13A assesses the implications of long term repeat exposure to BSO on intracellular GSH, showing GSH levels for each concentration of BSO given to cells, longitudinally, as a percent of control. All values for each group show a decrease in GSH from control in response to all concentrations of BSO; however the extent to which GSH decreases

varies between treatment groups as well as days of treatment. Figure 13B assesses the implications of long term repeat exposure to BSO on progesterone, showing progesterone levels for each concentration of BSO given to cells, longitudinally, as a percent of control. All cells show largely diminished levels of progesterone by 12 days of culture, maintained throughout 28 days. These findings indicate a relatively early reduction in GSH is followed later by reduced progesterone levels. However, this diminished progesterone appears to occur without strong correlation to the concentration of BSO. The data obtained are summarized in Tables 1 and 2.

Discussion

With aging, serum levels of testosterone typically are reduced [43, 73-76]. In Brown Norway rats as in men, reduced serum testosterone usually is not in response to reduced LH, but rather to a primary testicular defect. Thus, in the case of Brown Norway rats, Leydig cells isolated from the testes of aging rats produce less testosterone in response to luteinizing hormone (LH) than cells from young rats [73]. Although the cause(s) of reduced testosterone formation remains uncertain, it is known that aging cells, in general, can undergo deleterious changes in response to exposure to reactive oxygen-induced free radical reactions [53, 77-79]. Superoxide and other reactive oxygen species (ROS) are produced by the mitochondrial electron transport chain during oxidative phosphorylation [80]. In the case of Leydig cells, ROS are produced from this source, but in addition, Leydig cell cytochrome P450 enzymes catalyze the oxidation of metabolic intermediates in the steroidogenic pathway, thus generating additional free radicals [81]. Thus, mitochondrial superoxide production has been shown to increase in aging Leydig cells [14], and the antioxidant defense molecules superoxide dismutase-1 and -2, glutathione peroxidase, and GSH to decrease [12, 13]. Lipid peroxidation increases with age in Leydig [12] and adrenal cells [82], two of the major steroidogenically active cells in the body. It seems reasonable to conclude from these studies that imbalance in the oxidant/antioxidant environment of aging Leydig cells, perhaps by causing oxidative damage to proteins, lipids, and/or DNA [83],

might be the cause of reduced testosterone formation by these cells. However, these studies identified correlations, not cause-effect relationships.

Based upon a strong correlation between a diminished antioxidant response and cell aging, we made antioxidant depletion a focal point of the experiments that we describe herein [3, 12, 34, 61]. More specifically, a study indicating that GSH is an antioxidant significantly depleted in adult rat Leydig cells led us to investigate a possible correlation between GSH depletion, oxidative stress, and age-induced decline in steroid production. As such, the overarching objective of this thesis was to better understand the relationship between oxidative stress and Leydig cell aging, measured as a function of steroidogenic output.

Using MA-10 cells, we attempted to create a model for Leydig cell aging by manipulating the cell's redox environment to mimic that of an aged cell, and to determine if that would have an effect on steroidogenesis, either immediately or with a delayed onset. Specifically, the experiments concerning GSH depletion sought to test three different parameters concerning oxidative stress and cell function. The first parameter concerned increasing the oxidant load of MA-10 cells by introducing a stressor (t-BuOOH). The second concerned increasing the oxidant load while reducing the ability of the cell to respond to stress by reducing intracellular GSH (t-BuOOH with BSO). The third concerned reducing the ability of the cell to deal with intracellular stress over an extended period of time (BSO over time). We hypothesized that following GSH depletion, MA-10 cells would be highly susceptible to oxidative stress induced by t-BuOOH and exhibit diminished steroid synthesis; and that long-term GSH depletion, even in the absence of a stressor, would precede a decline in steroid synthesis, similar to what has been observed to occur *in vivo*.

In order to induce oxidative stress and alter the Leydig cell redox environment to an extent similar to that seen in aged cells, MA-10 cells were treated with BSO to reduce GSH. The 24 hour treatment of MA-10 cells with BSO demonstrated that BSO causes an immediate, though small, reduction in intracellular GSH levels and thus is capable of altering a cell's redox

environment. This relatively rapid GSH depletion had little to no effect on steroidogenesis. To determine whether an altered intracellular GSH environment affected the susceptibility of the cells to oxidative stress, MA-10 cells that had been incubated in BSO for 24 hours were then exposed to the stressor t-BuOOH. A rapid, concentration-dependent reduction in progesterone was seen. Interestingly, when control cells (cells not treated with BSO) were given increasing amounts of t-BuOOH, progesterone was found to decrease but to a significantly lesser extent than in cells pre-treated with BSO. This suggests that BSO pre-treatment exacerbated the decline in progesterone synthesis initiated by T-BuOOH alone.

At low concentrations of t-BuOOH (6.25 and 12.5 μM) there were relatively small differences observed between control and BSO pre-treated cells' ability to synthesize steroid. However at higher concentrations of t-BuOOH (50 and 100 μM), there was a marked decline in progesterone from BSO pre-treated cells in comparison to control cells. These results suggest that in a normal redox environment, with plentiful levels of GSH, the effects of free radical exposure to cells are minimal. However, in an environment with diminished levels of GSH, MA-10 cells become highly susceptible to free radical damage and, as a consequence, experience diminished steroid synthesis. This also implies that simply having an altered redox environment is not immediately sufficient to diminish steroid synthesis. Either an external stressor of some kind, and/or time, apparently are required for there to be an impact on the steroidogenic machinery of the cell. The inefficacy of BSO to have an immediate effect on steroidogenesis can be explained by the fact that cells contain pathways in addition to GSH that are also used for the removal of reactive oxygen species [57, 58]. In depleting GSH, these alternative pathways may be able to compensate for its absence and maintain a relatively healthy cell. When exposed to t-BuOOH, however, these GSH depleted cells might easily exhaust all other means of reactive oxygen species removal. These data suggest that there is a certain threshold of an altered redox environment wherein the effects of oxidative stress manifest in the form of reduced steroid synthesis.

While this is of great importance for understanding some of the factors involved in Leydig cell aging and decline, aging is of course a process that occurs over time and must be studied as such in order to be fully understood. To do so, further experiments were conducted to determine the effects of consistent GSH depletion by BSO over time, in the absence of the external stressor t-BuOOH. MA-10 cells were exposed to one of three concentrations of BSO (25, 50, or 100 μM) from 24 hours to 28 days and were assayed at 24 hours, 4 days, 8 days, 12 days and 28 days for both GSH and progesterone, the latter in response to maximal stimulation by LH. Consistent with the previous set of studies, after 24 hours of BSO, cells exhibited diminished GSH levels but no decline in progesterone. This persisted through days 4 and 8. By day 12, diminished levels of GSH were accompanied by a large decline in progesterone, and this continued through day 28. It is worth noting that while all cells given BSO exhibited diminished levels of GSH, there was little concentration-dependent correlation observed between the concentration of BSO given and concentration of GSH observed. Nevertheless, cells with diminished GSH concentrations still exhibited reduced progesterone synthesis, implying that over time, even a modestly altered anti-oxidant environment is able to hinder steroidogenesis.

These results are quite consistent with a set of studies concerning *Nrf2* knockout mice, which demonstrated that when Leydig cell antioxidant capacity is attenuated by knocking out *Nrf2*, it is eventually followed by a decrease in the ability to synthesize testosterone [15]. Taken together, these results fall in line with the free radical theory of aging, and further serve to provide evidence that Leydig cell senescence may indeed be induced by the accumulation of oxidative stress over a lifetime.

There are some curious observations, concerns, and limiting factors in the data. The extent to which GSH was depleted in the long-term BSO treated MA-10 cells differed in some respects with what occurs physiologically in aged cells. Leydig cells isolated from old Brown Norway rats have been shown to exhibit up to a 60% decrease in GSH in comparison to young cells, while BSO treated MA-10 cells showed decreases of up to only 40% [12]. These data

suggest, however, that even relatively modest GSH depletion may be sufficient to decrease steroidogenesis in cells over time. Also of note is the type of cell line used in these studies. MA-10 Leydig cells are a transformed tumor cell line, which turn over regularly and frequently. Theoretically, as cells are given fresh nutrients, their internal stores of proteins, lipids, and other important biomolecules should be restored each time they replicate. Therefore any damage incurred by reactive oxygen species to the aforementioned biomolecules might not manifest in subsequent generations unless direct damage to the cell's DNA is occurring. High susceptibility of the DNA region encoding the LH receptor to oxidative stress may be a factor in diminished steroidogenesis in spite of relative good health of the cell overall.

Another highly plausible scenario is that by treating these rapidly turning over cells with BSO, a known toxin, BSO resistant cells could have been selected for and otherwise normal MA-10 cells could have died. It is also possible that cells capable of functioning in spite of decreased GSH levels were selected for as well. Neither scenario is ideal given that these data are intended to reflect the responses of a typical Leydig cell.

Avenues to explore in the future studies include assaying long-term BSO treated cells for steroidogenic enzymes to determine the components of the steroidogenic pathway that are deficient and thus lead to reduced steroid synthesis. It would be of a particular significance if the step(s) in the pathway most affected by GSH depletion was LH-receptor coupling because this is the step affected in Leydig cells of the aging testis. Testing MA-10 cells for the ability to recover from long-term GSH depletion is of interest as well, specifically observing whether GSH and progesterone levels in these cells can be restored from BSO removal or even with the added presence of an inducer of GSH.

In summary, the results presented herein support the hypothesis that a cause-effect relationship exists between reduced antioxidant capacity after GSH depletion by BSO, and presumably increased reactive oxygen species (ROS). This, in turn, would expose Leydig cells, whether primary or MA-10 cells, to higher oxidative stress levels, which, over time, would result

in accelerated reductions in steroidogenic function. Indeed, such changes occur in vivo, over time, with aging in both rats and mice. The mechanisms by which loss of antioxidant capacity and increased ROS production occur in vivo remain uncertain.

Figures

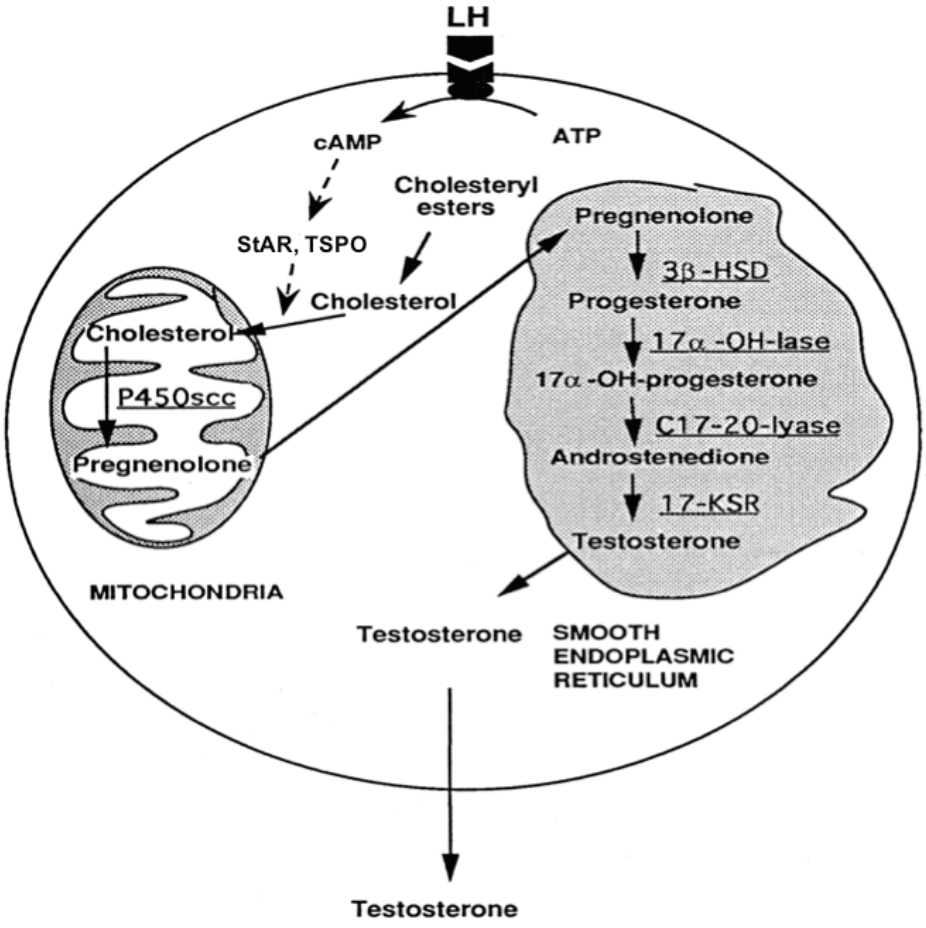

Figure 1: Illustration of the molecular events involved in testosterone production by Leydig cells. Luteinizing hormone (LH) binds to receptors on the Leydig cell plasma membrane, thereby initiating a cascade of events that includes increased intracellular cAMP formation, translocation of cholesterol into the mitochondria (with the involvement of steroidogenic acute regulatory protein and translocator protein), association of cholesterol with P450scc, production of pregnenolone from cholesterol in the mitochondria, translocation of pregnenolone from the mitochondria to the smooth endoplasmic reticulum, and conversion of pregnenolone to testosterone via a series of reactions in the smooth endoplasmic reticulum.

Zirkin, B.R. and H. Chen, *Regulation of Leydig Cell Steroidogenic Function During Aging.* Biology of Reproduction, 2000. 63(4): p. 977-981.

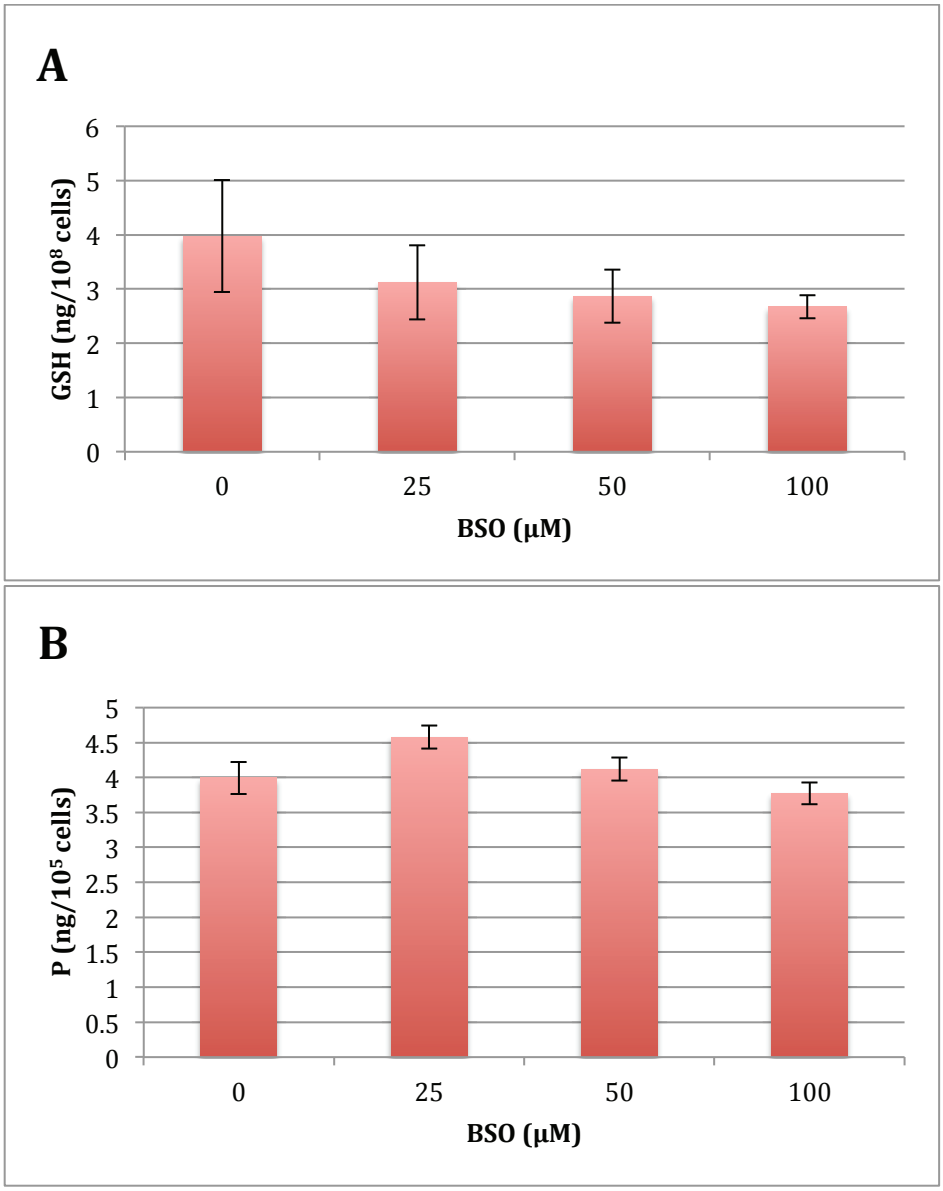

Figure 2: Effect of 24-hour BSO treatment on intracellular GSH and progesterone synthesis. Cells were treated with 0, 25, 50, or 100 µM BSO for a 24 hour period. Cells were then assayed for GSH (A). The remaining cells were stimulated with LH for 2 hours for progesterone assay (B).

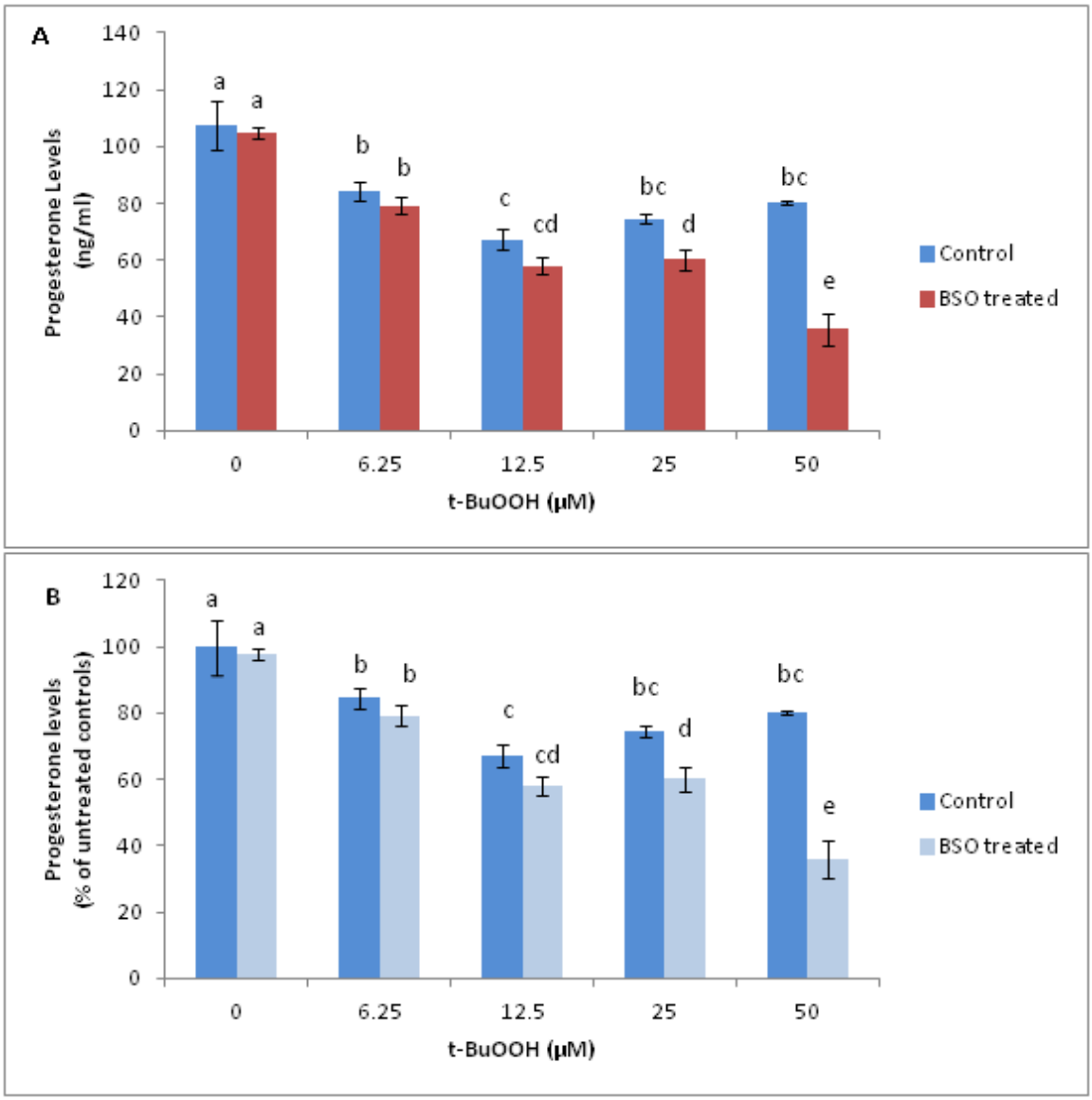

Figure 3: Effect of GSH depletion plus t-BuOOH on progesterone production by MA-10 Leydig Cells. The cells were treated with BSO alone for 24 hours then treated for an additional 2 hours with increasing concentrations of the oxidant t-BuOOH. Cells were then incubated with maximally stimulating LH and assayed for progesterone. Data are presented as progesterone in ng/ml (A) and as percent of control (B). Altering the redox environment of the cells with BSO plus t-BuOOH resulted in reduced progesterone, dependent upon t-BuOOH dose. (Figure taken from the Master's thesis of Keerti Balachandran)

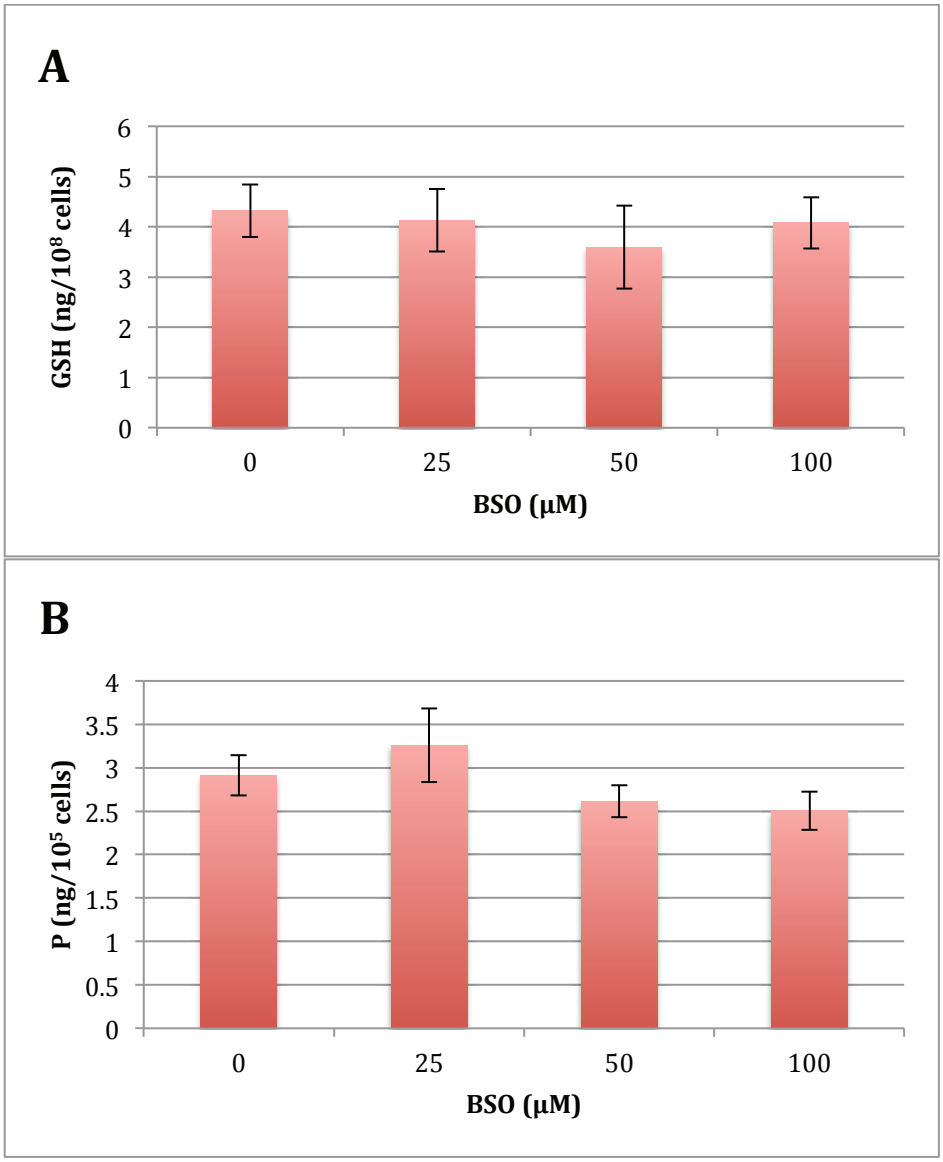

Figure 4: Effect BSO treatment on intracellular GSH and progesterone synthesis at 8 days culture. Cells were treated with 0, 25, 50, or 100 µM BSO for a 24 hour period. Cells were then assayed for GSH (A). The remaining cells were stimulated with LH for 2 hours for progesterone assay (B). Figures A and B show GSH and progesterone levels respectively on the 8th day of cell culture.

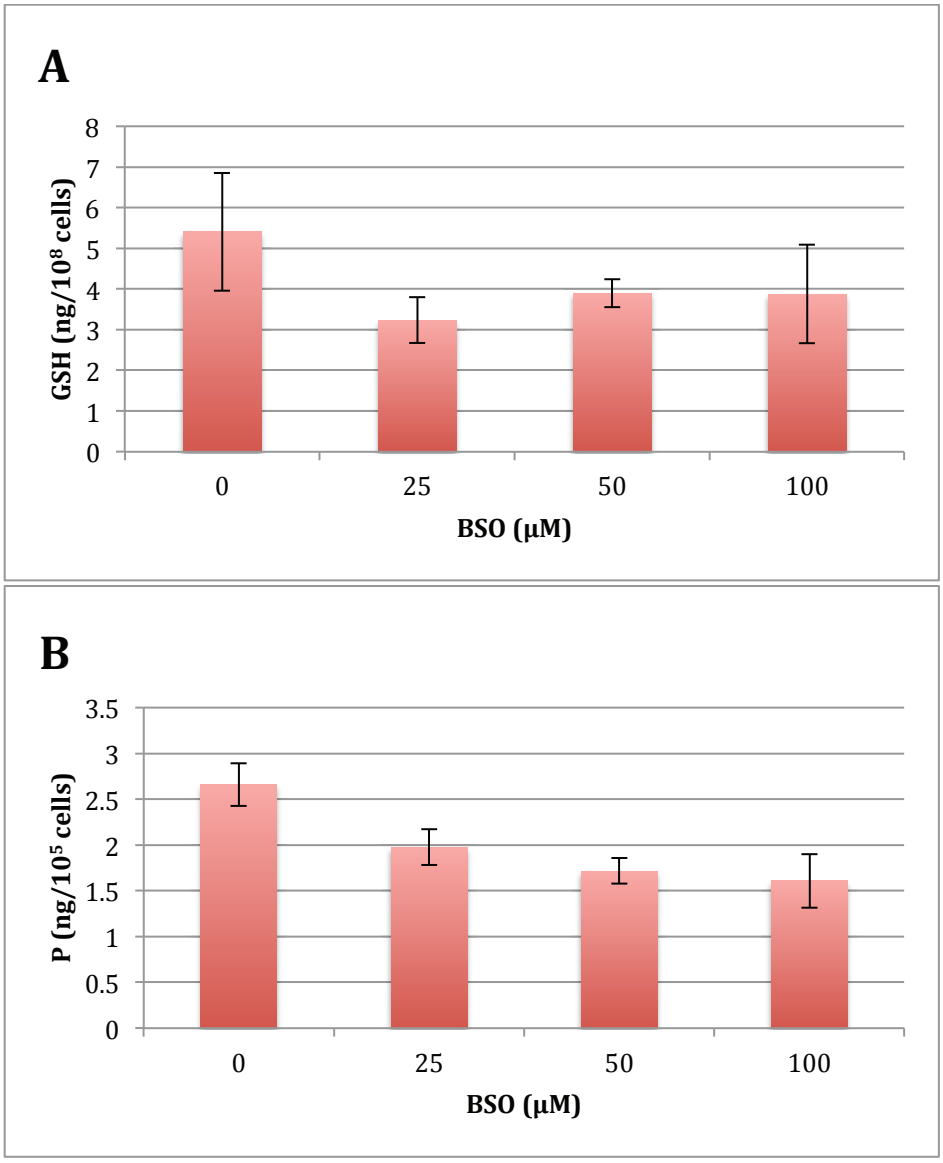

Figure 5: Effect of BSO treatment on intracellular GSH and progesterone synthesis at 12 days culture. Cells were treated with 0, 25, 50, or 100 µM BSO for a 24 hour period. Cells were then assayed for GSH (A). The remaining cells were stimulated with LH for 2 hours for progesterone assay (B). Figures A and B show GSH and progesterone levels respectively on the 12^{th} day of cell culture.

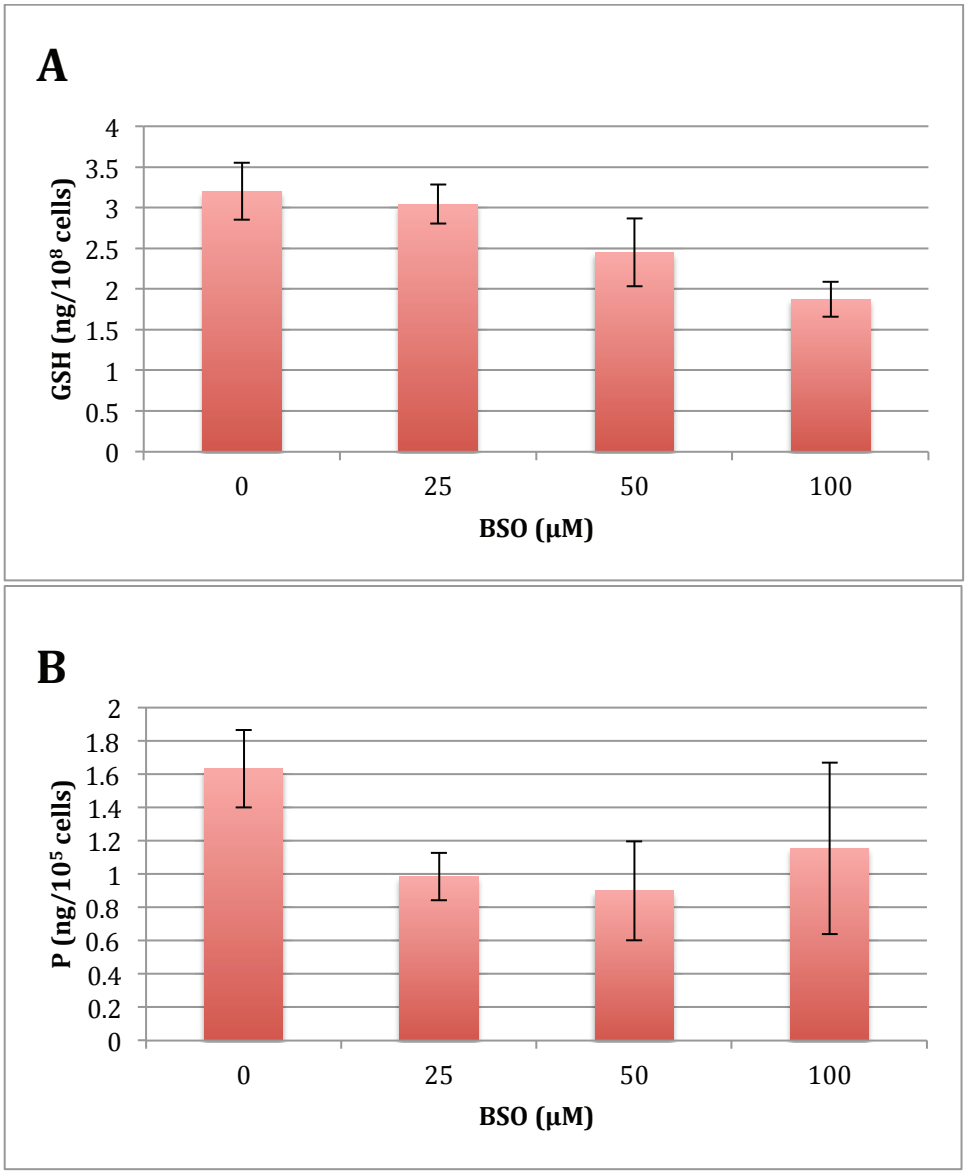

Figure 6: Effect of BSO treatment on intracellular GSH and progesterone synthesis at 28 days culture. Cells were treated with 0, 25, 50, or 100 µM BSO for a 24 hour period. Cells were then assayed for GSH (A). The remaining cells were stimulated with LH for 2 hours for progesterone assay (B). Figures A and B show GSH and progesterone levels respectively on the 28th day of cell culture.

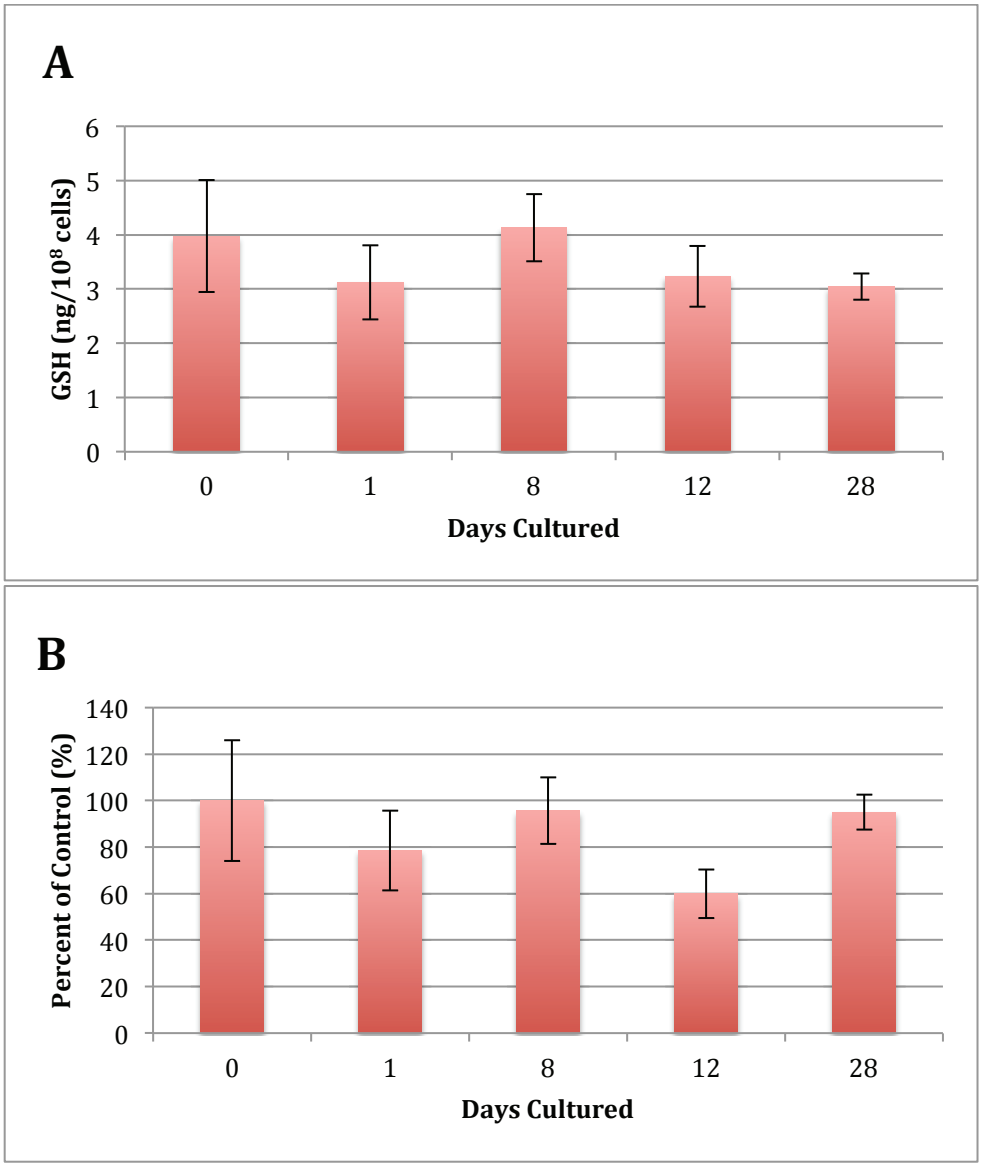

Figure 7: Effect of 25µM BSO incubation on GSH depletion over time. The cells were treated with 25 µM BSO for 24 hour period every 4 days for 28 days. Following BSO treatment, cells were assayed for GSH. Data are shown as ng GSH per 10^8 cells (A) and as percent of control cells given 0 µM BSO (B).

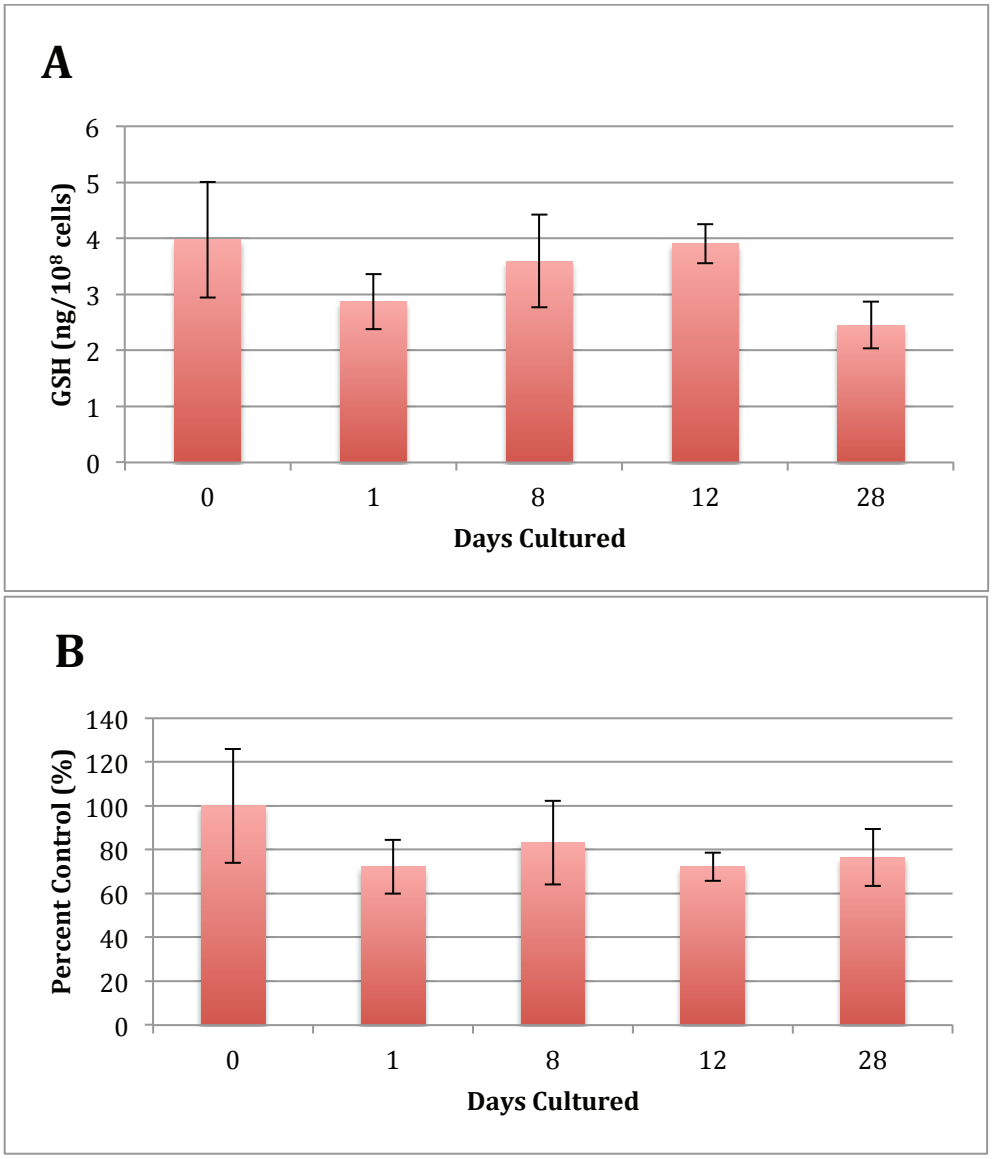

Figure 8: Effect of 50µM BSO incubation on GSH depletion over time. The cells were treated with 50 µM BSO for 24 hour period every 4 days for 28 days. Following BSO treatment, cells were assayed for GSH. Data are shown as ng GSH per 10^8 cells (A) and as percent of control cells given 0 µM BSO (B).

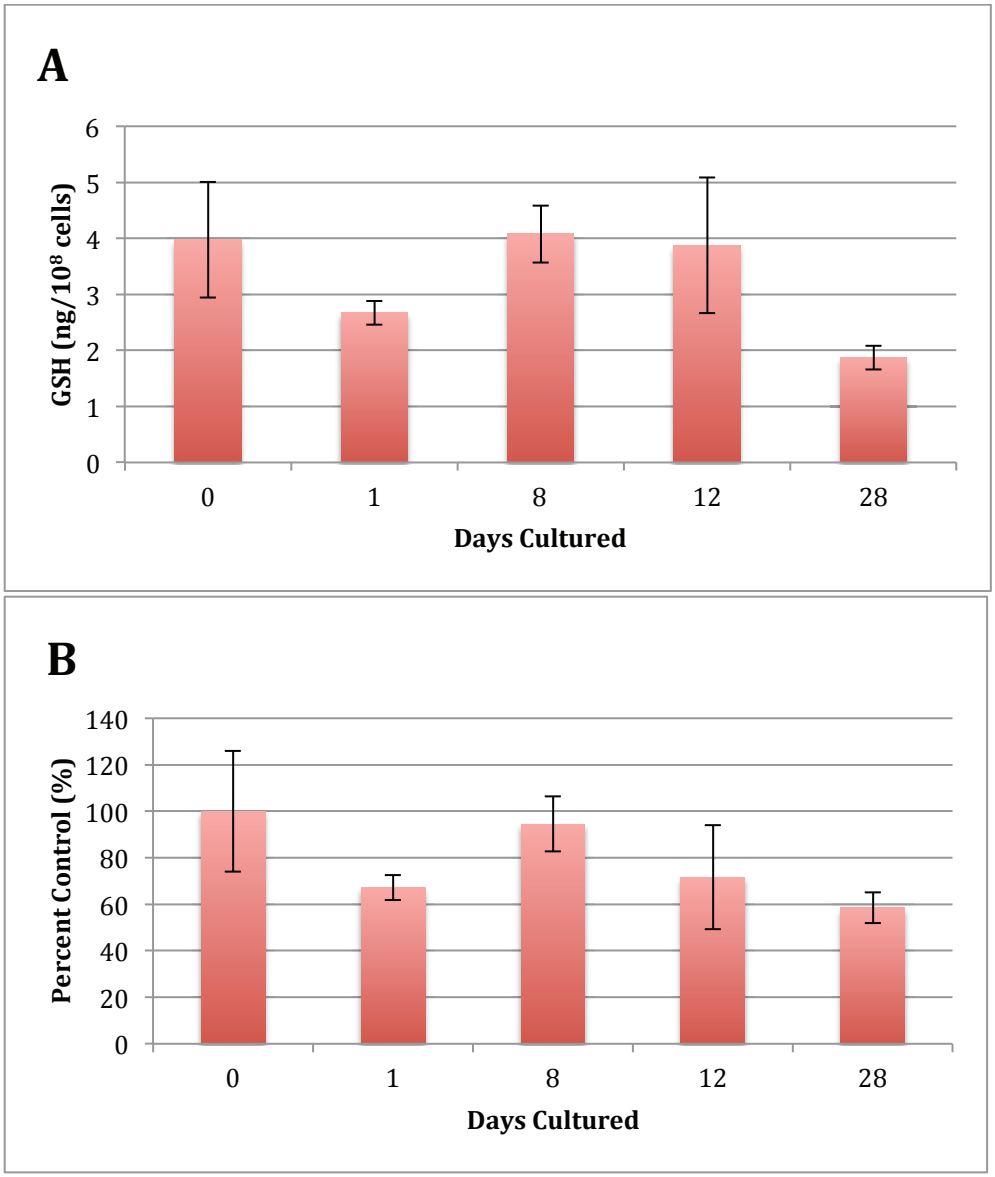

Figure 9: Effect of 100µM BSO incubation on GSH depletion over time. The cells were treated with 100 µM BSO for 24 hour period every 4 days for 28 days. Following BSO treatment, cells were assayed for GSH. Data are shown as ng GSH per 10^8 cells (A) and as percent of control cells given 0 µM BSO (B).

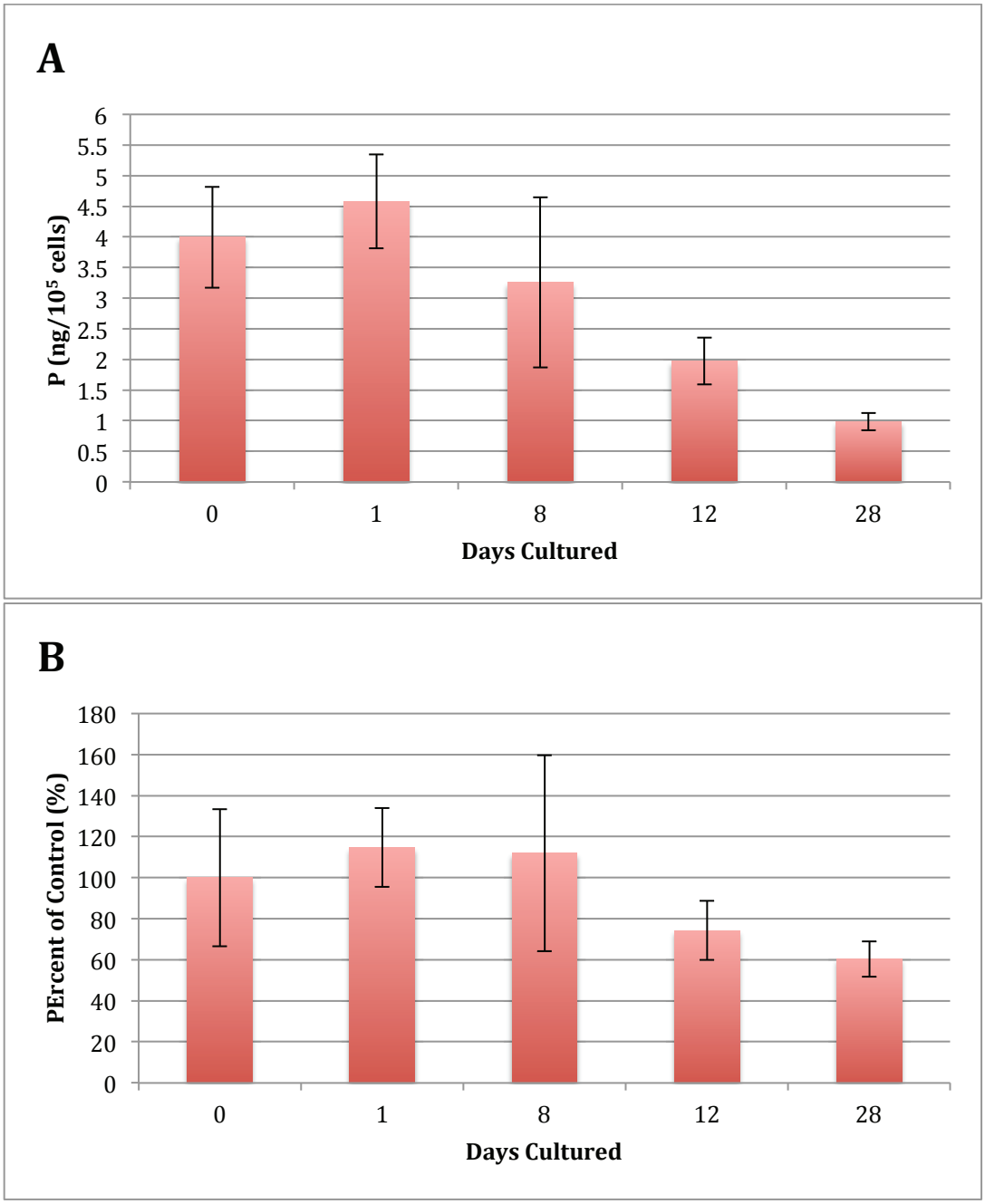

Figure 10: Effect of 25µM BSO incubation over time on progesterone synthesis. The cells were treated with 25 µM BSO for a 24 hour period every 4 days for 28 days. Cells were then washed, re-plated, and 24 hours later were stimulated with LH for 1 hour. Data are shown as ng of progesterone per 10^6 cells (A) and as percent of control cells given 0 µM BSO (B), cultured and assayed in conjunction with BSO treated cells.

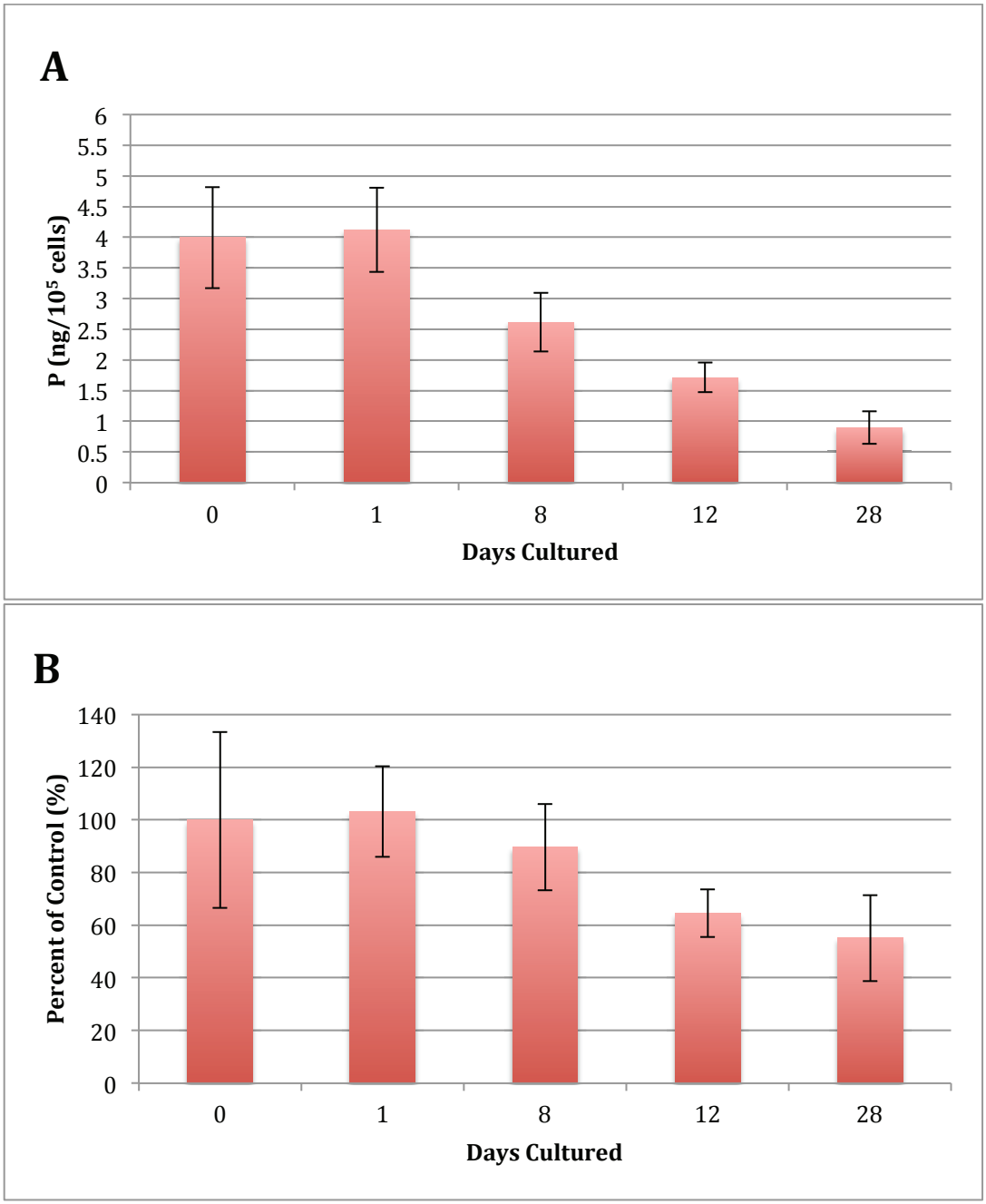

Figure 11: Effect of 50µM BSO incubation over time on progesterone synthesis. The cells were treated with 50 µM BSO for a 24 hour period every 4 days for 28 days. Cells were then washed, re-plated, and 24 hours later were stimulated with LH for 1 hour. Data are shown as ng of progesterone per 10^6 cells (A) and as percent of control cells given 0 µM BSO (B), cultured and assayed in conjunction with BSO treated cells.

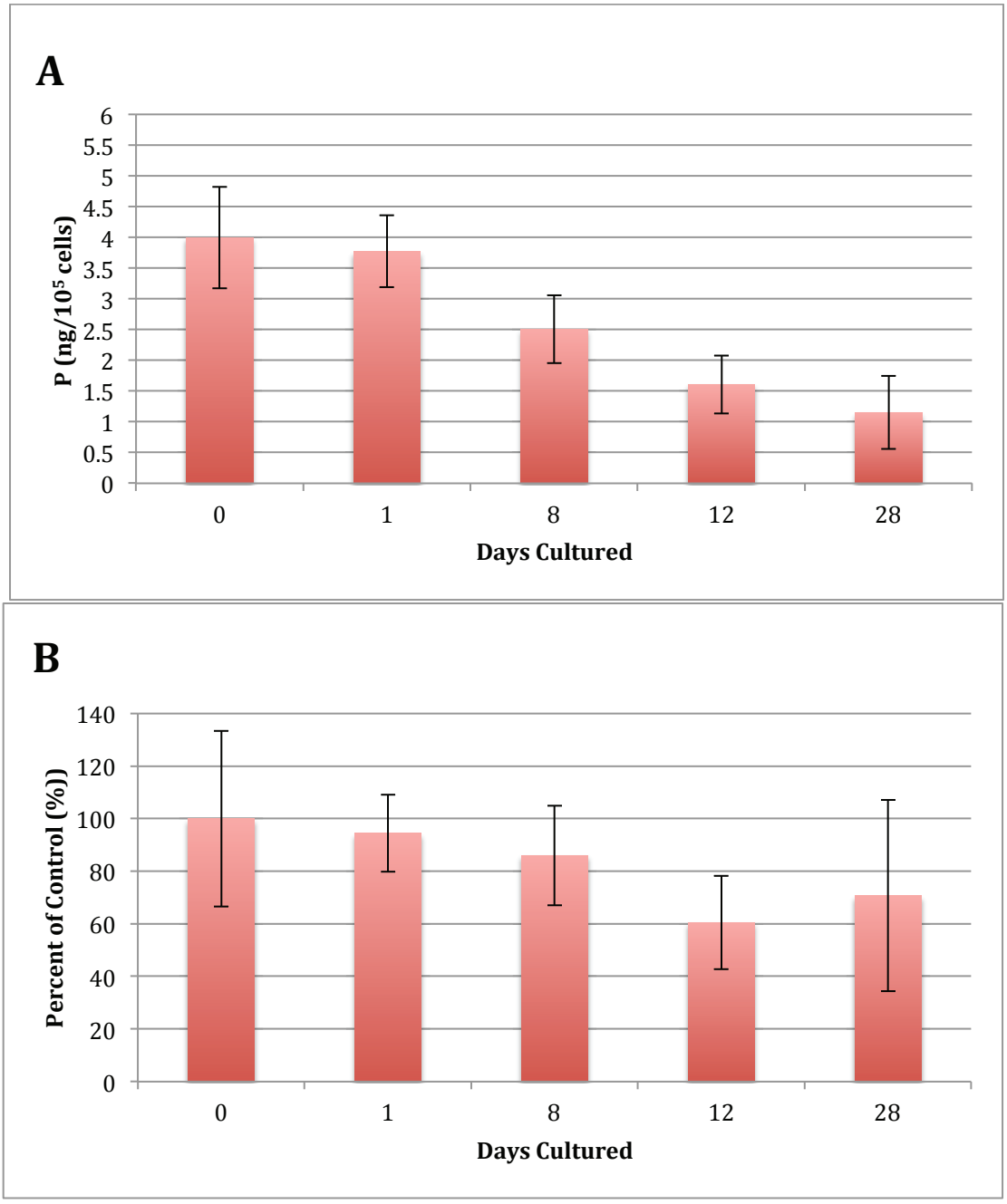

Figure 12: Effect of 100µM BSO incubation over time on progesterone synthesis. The cells were treated with 100 µM BSO for a 24 hour period every 4 days for 28 days. Cells were then washed, re-plated, and 24 hours later were stimulated with LH for 1 hour. Data are shown as ng of progesterone per 10^6 cells (A) and as percent of control cells given 0 µM BSO (B), cultured and assayed in conjunction with BSO treated cells.

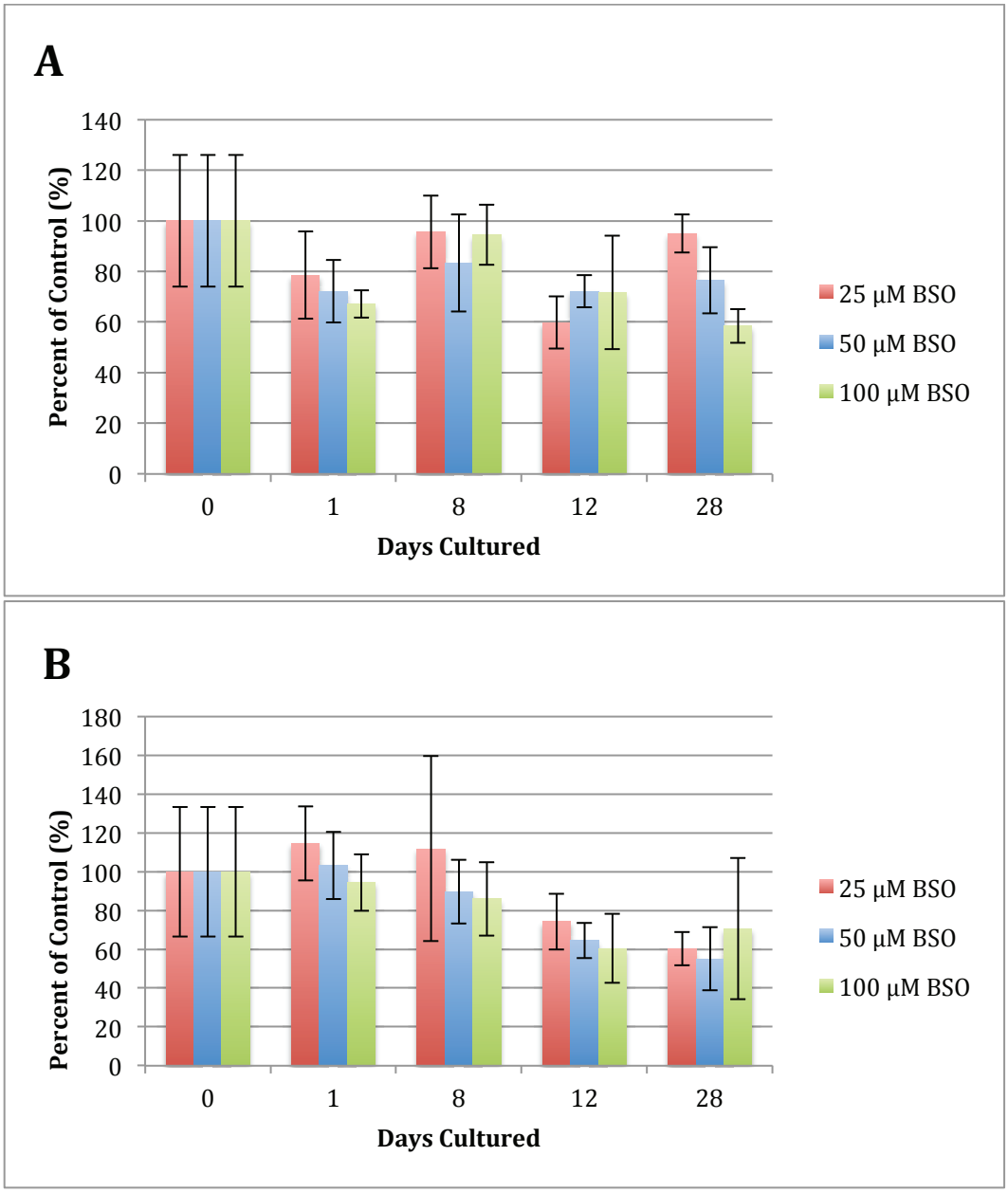

Figure 13: Effect of long-term (0-28 days) BSO incubation on intracellular GSH and progesterone as percent of control. Cells were cultured in M-199 culture medium in the presence of 0, 25, 50, or 100 µl/ml BSO, which was given for 24 hours in 4-day intervals. Following BSO treatment, cells were then washed, removed using trypsin, and assayed for GSH. Remaining cells not assayed were then re-plated, and 24 hours later were stimulated with LH for 1 hour for progesterone assay. Figures A and B show GSH and progesterone levels respectively throughout cell culture.

Days of Culture	0 µM BSO	25 µM BSO	50 µM BSO	100 µM BSO	P Value
1	3.975	3.124	2.869	2.671	0.549
8	4.315	4.129	3.595	4.078	0.871
12	5.404	3.235	3.901	3.874	0.496
28	3.201	3.043	2.451	1.873	0.034

Table 1: Intracellular GSH as a function of BSO concentration and days of cell culture.

Days of Culture	0 µM BSO	25 µM BSO	50 µM BSO	100 µM BSO	P Value
1	3.993	4.577	4.121	3.773	0.087
8	2.914	3.259	2.614	2.506	0.930
12	2.659	1.975	1.716	1.609	0.287
28	1.631	0.985	0.899	1.154	0.615

Table 2: Progesterone as a function of BSO concentration and days of cell culture.

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CURRICULUM VITAE

LANRE ADEKOLA
DOB: 06/22/1991
Minneapolis, MN

629 73rd Way N • Brooklyn Park, MN 55444 • (612) 598-5408• Ladekol1@gmail.com

EDUCATION

Johns Hopkins Bloomberg School of Public Health **Baltimore, MD**
- Master of Science in Biochemistry and Molecular Biology Expected Completion: **June 2015**

Johns Hopkins University **Baltimore, MD**
- Bachelor of Arts in Biology Completion: **May 2013**

EXPERIENCE

Johns Hopkins Krieger School of Arts & Sciences **Baltimore, MD**
Teaching Assistant **September-December 2014**
- Teaching assistant for Stem Cells and the Biology of Aging and disease course
- Graded exams, consolidating grades, and addressing student questions and concerns

Johns Hopkins Bloomberg School of Public Health **Baltimore, MD**
Research Assistant **Summer 2013**
- Worked under an NIH Merit Award Grant with the goal of developing new treatments for male infertility, hypogonadism, and health risks associated with aging.
- Studied the *in vitro* effects of cyclooxygenase-2 inhibitors and TSPO drug-ligands on Leydig cell testosterone synthesis.
- Cultured and treated mouse MA-10 cells with various drugs and measured effects on steroidogenesis via radioimmunoassay.

University of Minnesota St. Anthony Falls Hydraulic Laboratory **Minneapolis, MN**
Research Assistant **May 2008 to August 2012**
- As part of a research team, tested the efficacy of storm water detention ponds throughout the Twin Cities Metro Area.
- Conducted batch adsorption study to test potential of various agents to remove water pollutants via ion exchange chemistry.
- Using data analysis, determined that iron as well as various activated carbons were most efficient in removing pollutants.
- Research currently being implemented to develop newer, more efficient detention ponds.
- As part of research team, analyzed the effects of soil compaction on water infiltration.
- Measured hydraulic conductivity of soil in several high-risk areas across Twin Cities Metro Area.
- Using data analysis, determined soil in various parks was over-compacted, and contracted renovators to till soil and add compost.
- Conducted follow-up testing on the same areas a year later and infiltration drastically increased.